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(57) Abstract

The invention provides synthetic DNAs which are capable of expressing peptide proteins toxic to insects when incorporated within the genome of a suitable host organism, e.g. a baculovirus, the proteins being the same as or structurally and functionally similar to peptide toxins derived from molluscs, e.g. *Conus* spp.

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INDUCIBLE NITRIC OXIDE SYNTHASE AND GENE THEREFOR.

The invention relates to a novel nitric oxide (NO) synthase, DNA coding therefor, methods for detection of the NO synthase and the DNA, a method for screening for compounds capable of inhibiting or stimulating the NO synthase, and the compounds thereby identified.

The demonstration in 1987 of the formation of NO by an enzyme in vascular endothelial cells opened up what can now be considered as a new area of research (for review, see Moncada et al. (1991) Pharmacological Reviews, vol. 43, no. 2, 109-142). NO is synthesised from the amino acid L-arginine by the enzyme NO synthase.

The synthesis of NO from L-arginine by NO synthase is now recognized as an important pathway for regulating the function of a wide variety of cells and tissues. For example, in the blood vessel wall, NO is synthesised by the vascular endothelium to regulate smooth muscle tone and thus blood pressure. Nitric oxide synthase is also present in the central nervous system, where NO is a neurotransmitter/neuromodulator mediating the action of glutamate on NMDA receptors and mediating/modulating transmission in nerves previously recognised as nonadrenergic and noncholinergic. NO can also act as an autocrine regulator on some cells, including platelets, where it modulates their activation. NO generated by activated macrophages is also an important effector molecule in host defence. In this role, NO has been shown to possess anti-tumour and anti-microbial activity against various parasites in vitro and in vivo.

NO synthases can be classified into two types, namely inducible and constitutive NO synthases. NO synthase activity is constitutively expressed in a variety of cells including endothelial cells, neurons, platelets, adrenal gland cells, and endocardium cells. In contrast, NO synthase is inducible in macrophages, hepatocytes, Kupffer cells, vascular smooth muscle and vascular endothelium following activation with endotoxin and/or cytokines.

More recently, NO synthase has been shown to be induced in rabbit articular chondrocytes (Stadler et al. (1991) J. Immunol. 147, 3915-3920 and Palmer et al. (1992) Biochem. Biophys. Res. Commun. 188, 209-215). We have observed that in human chondrocytes the enzyme is induced by interleukin-1β (IL-1β). The induction of NO synthase in human cells has previously only been reliably shown in hepatocytes (Nussler et al (1992), J. Exp. Med. 176, 261-264). The induction of NO synthase in chondrocytes is likely to have a role in joint disease (i.e. arthritic disease). IL-1β concentrations are increased in the inflamed joint and under these conditions NO synthase is likely to be induced.

The NO synthase enzymes comprise a family that can be distinguished on the basis of comparative DNA sequence analysis. Sequences have been reported for the constitutive neuronal NO synthase cDNAs from rat and man (Bredt et al. (1991) Nature 351, 714-718 and Nakane et al. (1993) Febs. Lett. 316, 175-180), the constitutive endothelial NO synthase cDNAs from bovine and human tissue (Lamas et al. (1992) Proc. Natl. Acad. Sci. USA 89, 6348-6352; Janssens et al. (1992) J. Biol Chem. 267, 14519-14522; Sessa et al. (1992) J. Biol. Chem. 267, 15274-15276; Marsden et al. (1992) Febs. Lett. 307, 287-293) and an inducible NO synthase cDNA from a rodent macrophage line (Lyons et al. (1992) J. Biol. Chem. 267, 6370-6374; Xie et al. (1992) Science 256, 225-228; and Lowenstein et al. (1992) Proc. Natl. Acad. Sci. USA 89, 6711-6715). A comparison of the deduced protein sequences derived from the three classes of NO synthase enzymes shows, overall, approximately 50-60% similarly. For all three enzymes, the highest degree of similarity occurs around a conserved region within the first third of the molecule, thought to represent the L-arginine binding site.

Although two distinct forms of the constitutive enzyme have been described at the molecular level, with high identity between the rat, and human neuronal forms and between the bovine and human endothelial forms, only the murine

macrophage form of the inducible NO synthase has been similarly characterized.

In the present invention, a novel human inducible NO synthase has been characterized. The full length cDNA encoding the NO synthase has been cloned and expressed using human articular chondrocytes activated with IL-1β. The NO synthase enzyme has utility in a number of settings as described hereinafter, but is of particular value in a research environment in which it can be used in a screen or assay to identify compounds that are capable of inhibiting or stimulating the activity of the enzyme. Such compounds have utility in the clinic for the treatment of indications as described hereinafter.

The present invention provides an NO synthase having the sequence of SEQ ID NO: 2, or an NO synthase having a sequence at least 85% identical to the sequence of SEQ ID NO: 2. The NO synthase may, for example, have a sequence at least 90%, at least 95%, at least 98% or at least 99% identical to SEQ ID NO: 2. The NO synthase is generally of human origin, preferably human chondrocyte origin.

The expression 'NO synthase' includes the full length cDNA encoding the NO synthase and any fragment thereof. The fragment of the NO synthase may be a fragment at least 6 amino acids in length. The length of the fragment may be, for example, at least 8, at least 12, at least 24, at least 48 or at least 96 amino acids, and is intended to include the fragment corresponding to the active domain of the enzyme,i.e. the domain which catalyses the synthesis of NO from L-arginine.

The NO synthase is generally in substantially pure form. Preferably, the NO synthase in substantially pure form comprises a preparation in which at least 90%, at least 95%, at least 98% or at least 99% of the weight of protein in the preparation is the NO synthase of the invention.

The NO synthase will usually be obtained by recombinant DNA techniques. However, the NO synthase may be obtained using biochemical purification of the protein from its

natural origin.

The invention provides a DNA molecule encoding the NO synthase. The DNA molecule may contain the sequence of nucleotides 226 to 3687 of SEQ ID NO: 1. Alternatively, the DNA molecule may contain a sequence at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to the sequence of nucleotides 226 to 3687 of SEQ ID NO: 1.

A person of ordinary skill in the art would know how to obtain a DNA molecule according to the invention using the sequences disclosed herein, without undue experimentation. The DNA molecules according to the invention could be produced by various means, such as, for example, DNA synthesis, or more preferably, by recombinant DNA techniques. Techniques for synthesising DNA molecules are described by, for example, Wu et al (Prog. Nucl. Acid. Res. Molec. Biol. 21, 101-141 (1978)). Techniques for constructing recombinant molecules are described by Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

The present NO synthase may be obtained by making a library of replicable expression vectors. The library may be created by cloning genomic DNA or, more preferably, cDNA into a parent vector. The cDNA may be obtained using the poly A^+ mRNA of a cell (e.g. a chondrocyte) in which NO synthase production has been induced, e.g. by a cytokine such as IL-1β. The library is screened for members containing the desired nucleic acid sequence, e.g. by means of a DNA probe or antibody. A probe having a sequence identical to a portion of the sequence of nucleotides 226 to 3687 of SEQ ID NO: 1 or exactly complementary to a portion of the sequence nucleotides 226 to 3687 of SEQ ID NO: 1 may be used to identify an NO synthase coding sequence not identical to the sequence of nucleotides 226 to 3687 of SEQ ID NO: 1 (e.g. from 85% to 99% identical to nucleotides 226 to 3687 of SEQ ID NO: 1) by low stringency hybridization.

A replicable expression vector is a vector which

contains the appropriate origin of replication sequence for replication of the vector and the appropriate sequences for expression of the foreign nucleotide sequence in the vector. The sequences for expression of a foreign sequence will generally include a transcription promoter operably linked to the foreign sequence. The term "operably linked" refers to a linkage in which the promoter and foreign sequence are connected in such a way to permit expression of the foreign sequence. The transcription promoter sequence may be part of the parent vector sequence into which the foreign sequence is inserted. Alternatively, the promoter sequence may be a native promoter sequence of a gene encoding an NO synthase of the invention, so that the NO synthase is inducible by IL-1β. A vector may be, for example, a plasmid, virus or phage vector. A vector may contain one or more selectable markers, for example an ampicillin resistance gene in the case of a bacterial vector or a neomycin resistance gene in the case of a mammalian vector. A foreign gene sequence inserted into a vector may be transcribed in vitro or the vector may be used to transform or transfect a host cell.

According to one embodiment of the invention, there is provided a host cell transformed or transfected with a vector. A vector and host cell will be chosen so as to be compatible with each other, and may be prokaryotic or eukaryotic. A prokaryotic host may, for example, be E. coli, in which case the vector may, for example, be a bacterial plasmid or a phage vector. A eukaryotic host may, for example, be a yeast (e.g. S. cerevisiae) a chinese hamster ovary (CHO) cell or an insect cell (e.g. Spodoptera frugiperda). When the host is an insect cell, the vector is generally a baculovirus vector (reviewed by Luckow and Summers (1988) in BIO/TECHNOLOGY, Vol. 6, 47-55).

An NO synthase according to the invention may be produced by a method comprising

(a) culturing a host cell under conditions in which the cell expresses the NO synthase; and

(b) recovering the NO synthase from the culture.

In this method, expression of NO synthase may be induced by a cytokine such as IL-1β. The NO synthase may be recovered from either the host cell or, where the NO synthase is secreted by the host cell, from the culture supernatant.

The invention includes a an oligonucleotide fragment having a sequence of a portion of a DNA molecule encoding an NO synthase of the invention, and an oligonucleotide fragment having a sequence at least 85%, at least 90%, at least 95%, at least 98%, or at least 99%, identical to a sequence encoding an NO synthase of the invention or fragment thereof. The fragment is at least 12 nucleotides in length, e.g. at least 15, at least 18, at least 30, at least 60, at least 180, or at least 720 nucleotides in length. The fragment may be single or double stranded. When the fragment is single stranded, it may have a sequence from either a sense or antisense strand of a DNA molecule encoding an NO synthase of the invention. An antisense fragment may be useful in the therapeutic treatment of a disease involving over-expression of NO synthase, whilst a full-length expression fragment may be of use in treatment of conditions requiring stimulation of the NO synthase, for example treatment of some viral diseases or solid tumours.

The fragment will generally be DNA, although other types of nucleic acid may be used, for example RNA or modified DNA. A number of different types of nucleic acid modification are known in the art. These include methylphosphonate and phosphorothioate backbones, and addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule.

The oligonucleotide fragment may be an oligonucleotide probe or an oligonucleotide polymerase chain reaction (PCR) primer, which will hybridise to a nucleic acid molecule (e.g. a DNA or RNA molecule) encoding an NO synthase of the invention. The probe or a pair of primers may be used to detect or quantitatively determine the nucleic acid sequence. This has diagnostic utility in detecting and quantitatively determining NO synthase mRNA associated with a

disease state, for example arthritic disease, high blood pressure, disorders of the central nervous system, and cancers such as breast cancer.

A fragment which is a probe or primer may carry a revealing label, such as ^{32}P, digoxigenin or biotin. Preferably, the probe or primer will specifically hybridise only to its target sequence, e.g. a portion of SEQ ID NO: 1, and not to other sequences. However, it will be appreciated that this will not always be the case, and the probe or primer may only be selective for its target sequence. A probe or primer which hybridises only to its target sequence will generally be exactly complementary to the target sequence (e.g. a portion of the sequence of nucleotides 226 to 3687 of SEQ ID NO: 1) whereas a probe or primer which is only selective may have a sequence which is, for example, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% complementary to the target sequence. A probe which is not exactly complementary to its target sequence has utility in the identification of new NO synthase nucleotide sequences having a sequence similar to the sequence of nucleotides 226 to 3687 of SEQ ID NO: 1. A fragment which is a probe or primer may have from 12 to 60 nucleotides, e.g. from 12 to 40 nucleotides, or from 15 to 30 nucleotides.

Primers for PCR are generally provided as a pair. A first primer hybridises to a sense sequence 3' to the sequence to be amplified and a second primer hybridises to an antisense sequence 5' to the sequence to be amplified. This allows synthesis of double stranded DNA representing the region between the two primers.

Thus, the invention includes a method of amplifying a target nucleic acid sequence present in a nucleic acid encoding an NO synthase of the invention, which method comprises carrying out PCR employing a primer of the invention. Such a method generally comprises carrying out cycles of

(a) denaturing double stranded DNA containing the target sequence to obtain single stranded DNA;

(b) hybridizing a first primer to a sense strand 3' to the target sequence, and hybridising a second primer to an antisense strand 5' to the target sequence; and

(c) synthesising DNA from the first and second primers.

The number of cycles is suitably from 10 to 50, preferably 20 to 40, more preferably 25 to 35. The method may be carried out starting from a double stranded nucleic acid (e.g. dsDNA) or a single stranded nucleic acid (e.g. mRNA). The target sequence may be a complete NO synthase encoding sequence or a partial NO synthase encoding sequence.

As will be appreciated by a person skilled in the art, the method described above is based upon the well-known polymerase chain reaction (PCR) method. A skilled person would know of detailed protocols for carrying out PCR and reverse transcriptase-PCR (RT-PCR). Reviews of PCR are provided by Mullis (1986) Cold Spring Harbor Symp. Quant. Biol. 51, 263-273; Saiki et al. (1985) Bio/Technology 3, 1008-1012; and Mullis et al (1987) Meth. Enzymol 155, 335-350.

An oligonucleotide probe according to the invention has utility in detecting or quantitatively determining a nucleic acid (e.g. a DNA or RNA) encoding an NO synthase according to the invention. Conventional methods for detecting or quantitatively determining a nucleic acid may be used, for example in situ hybridization, Southern blotting or Northern blotting. Accordingly, there is provided a method of detecting or quantitatively determining in a sample a target nucleic acid sequence present in a nucleic acid encoding an NO synthase according to the invention, which method comprises

(a) contacting the probe with the sample; and

(b) detecting or quantitatively determining hybridization of the probe with any target nucleic acid sequence present in the sample.

The sample containing the target nucleotide sequence may, for example, be a tissue specimen, a tissue extract or

cell extract from a patient suffering from a disease associated with abnormal NO synthase activity such as arthritis, high blood pressure, a disorder of the central nervous system, or a cancer such as breast cancer. Alternatively, the sample may, for example, be a sample produced as a result of a recombinant DNA procedure, in which case the sample may be a vector or an extract of host cells. The target nucleic acid sequence may be a complete NO synthase encoding sequence or a partial NO synthase encoding sequence.

A preferred method of detecting or quantitatively determining a target nucleic acid sequence in a sample comprises

(i) subjecting the sample to gel electrophoresis to separate the nucleic acids;

(ii) transferring the separated nucleic acids onto a solid support (e.g. a nitrocellulose support) by blotting; and

(iii) hybridising a probe according to the invention to the target nucleic acid sequence.

A probe can be used in an <u>in situ</u> hybridization procedure to locate a nucleic acid sequence encoding an NO synthase of the invention. This can be done to determine the spatial distribution of NO synthase encoding DNA or mRNA sequences in a cell or tissue. In the case of mRNA detection, the tissue is gently fixed so that its RNA is retained in an exposed form and the tissue is then incubated with a labelled complementary probe.

A polypeptide fragment of the present invention has utility in, for example, producing antibodies against an NO synthase.

Thus, the invention includes an antibody specific for an NO synthase according to the invention. The antibody has utility in detecting and quantitatively determining NO synthases, and hence is useful in diagnosis of diseases associated with NO synthase, such as the diseases listed herein. An antibody of the present invention may also be of use

in identifying which NO synthase enzyme is responsible for a particular condition. The antibody also has utility in production of NO synthases by recombinant DNA procedures, for example in detection of positive clones containing a target sequence. Furthermore, the antibody may be of use as a therapeutic agent.

The antibody is preferably monoclonal, but may also be polyclonal. The antibody may be labelled. Examples of suitable antibody labels include radiolabels, biotin (which may be detected by avidin or streptavidin conjugated to peroxidase), alkaline phosphatase and fluorescent labels (e.g. fluorescein and rhodamine). The term "antibody" is used herein to include both complete antibody molecules and fragments thereof. Preferred fragments contain at least one antigen binding site, such as Fab and $F(ab')_2$ fragments. Humanised and chimaeric antibodies and fragments thereof are also included within the term "antibody".

The antibody is produced by raising antibody in a host animal against an NO synthase according to the invention or an antigenic epitope thereof (hereinafter "the immunogen"). Methods of producing monoclonal and polyclonal antibodies are well-known. A method for producing a polyclonal antibody comprises immunising a suitable host animal, for example an experimental animal, with the immunogen and isolating immunoglobulins from the serum. The animal may therefore be inoculated with the immunogen, blood subsequently removed from the animal and the IgG fraction purified. A method for producing a monoclonal antibody comprises immortalising cells which produce the desired antibody. Hybridoma cells may be produced by fusing spleen cells from an inoculated experimental animal with tumour cells (Kohler and Milstein, Nature 256, 495-497, 1975).

An immortalized cell producing the desired antibody may be selected by a conventional procedure. The hybridomas may be grown in culture or injected intraperitoneally for formation of ascites fluid or into the blood stream of an

allogenic host or immunocompromised host. Human antibody may be prepared by in vitro immunisation of human lymphocytes, followed by transformation of the lymphocytes with Epstein-Barr virus.

For the production of both monoclonal and polyclonal antibodies, the experimental animal is suitably a goat, rabbit, rat or mouse. If desired, the immunogen may be administered as a conjugate in which the immunogen is coupled, for example via a side chain of one of the amino acid residues, to a suitable carrier. The carrier molecule is typically a physiologically acceptable carrier. The antibody obtained may be isolated and, if desired, purified.

The invention includes a method of detecting or quantitatively determining in a sample an NO synthase of the invention, which method comprises

(a) contacting the sample with an antibody of the invention; and

(b) detecting or quantitatively determining the binding of the antibody.

A preferred method for detecting or quantitatively determining an NO synthase is Western blotting. Such a method can comprise the steps of

(i) subjecting a sample containing a target NO synthase to gel electrophoresis to separate the proteins in the sample;

(ii) transferring the separated proteins onto a solid support (e.g. a nitrocellulose support) by blotting; and

(iii) allowing an antibody according to the invention which has been labelled to bind to the target NO synthase.

Preferred methods of quantitative determination are ELISA (enzyme-linked immunoassay) methods such as a non-competitive ELISA methods. Typically, an ELISA method comprises the steps of

(i) immobilising on a solid support an unlabelled

antibody according to the invention;

(ii) adding a sample containing the target NO synthase such that the NO synthase is captured by the unlabelled antibody;

(iii) adding an antibody according to the invention which has been labelled; and

(iv) quantitatively determining the amount of bound labelled antibody.

An antibody of the invention may be employed histologically for _in situ_ detection of an NO synthase, e.g. by immunofluorescence or immunoelectron micropsy. _In situ_ detection may be accomplished by removing a histological specimen from a patient, and allowing a labelled antibody to bind to the specimen. Through use of such a procedure, it is possible to determine not only the presence of an NO synthase but also its distribution.

An antibody of the invention may be used to purify a target NO synthase. Conventional methods of purifying an antigen using an antibody may be used. Such methods include immunoprecipitation and immunoaffinity column methods. In an immunoaffinity column method, an antibody in accordance with the invention is coupled to the inert matrix of the column and a sample containing the target NO synthase is passed down the column, such that the target NO synthase is retained. The NO synthase is then eluted.

The sample containing the target NO synthase used in the detection, determination and purification methods may be a tissue specimen, a tissue extract or a cell extract from a patient suffering from a disease associated with NO synthase, such as a disease listed above. Alternatively, the sample may be one produced as a result of recombinant DNA procedures, e.g. a vector or an extract of host cells.

An NO synthase of the invention is useful for screening for substrates which inhibit or stimulate the enzyme. The invention includes a method for identifying a substrate which inhibits or stimulates the NO synthase, which method

comprises

(a) incubating the NO synthase with the substrate;

(b) measuring the activity of the NO synthase; and

(c) comparing the activity measured in (b) above with the activity of the NO synthase in the absence of the substrate.

For example, the activity of a substrate as an inhibitor or stimulator of the NO synthase of the present invention can be determined by an assay in which activated chondrocytes are incubated with the substrate, and NO synthase activity recorded using a dual wavelength spectrophotometer, reading at 401 and 421.

The invention also extends to substrates identified by the use of a screen hereinbefore described. Preferably the substrate is a chemical molecule of relatively low molecular weight, for example, less than about 1000. Examples of suitable classes of molecule include arginine analogues and isothiourea derivatives. Alternatively, the substrate can be a macromolecule, for example an antibody.

The invention also includes an enzyme-substrate complex which comprises an NO synthase as described herein and a substrate capable of inhibiting or stimulating the activity of the NO synthase. The enzyme-substrate complex optionally exists in an <u>ex vivo</u> situation.

A substrate which inhibits or stimulates the NO synthase enzyme is of utility in medical therapy. Inhibition of the inducible NO synthase may have many clinical utilities, for example in the treatment of septic shock and in particular in the treatment of hypotension assosiated therewith, in therapy with cytokines such as TNF, IL-1 and IL-2 or therapy with cytokine-inducing agents, for example 5, 6-dimethylxanthenone acetic acid, as an adjuvant to short term immunosuppression in transplant therapy, in patients suffering from inflammatory conditions in which an excess of NO contributes to the pathophysiology of the condition, for example adult respiratory distress syndrome and myocarditis, and in autoimmune and/or

inflammatory conditions such as arthritis and rheumatoid arthritis. Inhibition of the NO synthase enzyme may also be of use in the treatment of cerebral ischemia, CNS trauma, epilepsy, AIDS dementia, chronic neurodegenerative disease and chronic pain, and conditions in which non-adrenergic non-cholinergic nerve may be implicated such as priapism, obesity and hyperphagia. On the other hand, stimulation of the inducible NO synthase would lead to increased NO levels in the body and may be of use in treating parasitic and/or viral diseases, and killing tumour cells.

There is also provided within the scope of the present invention a pharmaceutical formulation which comprises one or more of a substrate identified using the present invention, an NO synthase as described herein, or an antibody of the present invention in combination with a pharmaceutically acceptable carrier or diluent therefor, and optionally one or more further therapeutic agents.

Formulations comprising a substrate, include those suitable for oral, parenteral (including subcutaneous, intradermal, intramuscular, intravenous and intraarticular), rectal and topical (including dermal, buccal, sublingual and intraocular) administration although the most suitable route may depend upon for example the condition and disorder of the recipient. Formulations comprising an NO synthase as described herein, or an antibody are those suitable for parenteral administration. All formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing into association a substrate, an NO synthase as described herein, or an antibody ("active ingredient") with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both and then, if necessary, shaping the product into the desired formulation.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

Formulations for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example, saline, water-for-injection, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Formulations for rectal administration may be presented as a suppository with the usual carriers such as cocoa butter or polyethylene glycol.

Formulations for topical administration in the mouth, for example buccally or sublingually, include lozenges comprising the active ingredient in a flavoured basis such as sucrose and acacia or tragacanth, and pastilles comprising the active ingredient in a basis such as gelatin and glycerin or sucrose and acacia.

Preferred unit dosage formulations are those containing an effective dose, as hereinbelow recited, or an appropriate fraction thereof, of the active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of

this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

The substrates are preferably administered orally or via injection at a dose of from 0.1 to 500mg/kg per day. The dose range for adult humans is generally from 5mg to 35g/day and preferably 5mg to 2g/day. Tablets or other forms of presentation provided in discrete units may conveniently contain an amount of compound of the invention which is effective at such dosage or as a multiple of the same, for instance, units containing 5mg to 500mg, usually around 10mg to 200mg.

The NO synthase as described herein or antibody are administered parenterally at a dose of from 1 to about 100 mg for an adult patient, preferably 1 - 10 mg, usually administered daily for a period between 1 and 30 days. A two part dosing regime may be preferable wherein 1 - 5 mg are administered for 5 - 10 days followed by 6 - 15mg for a further 5 - 10 days.

The precise amount of active ingredient administered to a patient will be the responsibility of the attendant physician. However the dose employed will depend on a number of factors, including the age and sex of the patient, the precise disorder being treated, and its severity. Also the route of administration may vary depending on the condition and its severity.

The present invention will now be described by way of example only, and is not intended to be limiting thereof.

EXAMPLE 1

Description of the Drawings

Figure 1 shows the results of Northern blot analysis of inducible NO synthase specific mRNA from human chondrocytes. $PolyA^+$ mRNA (0.25 μg), extracted from induced and uninduced cells, was electrophoresed through a formaldehyde-agarose gel

and transferred to a nylon membrane. The blot was hybridized with a full-length cDNA probe labelled with digoxigenin and washed under high stringency conditions. A positively hybridizing band at 4.4 kb) is apparent only in track 2 (induced). Track 1 was loaded with the same amount of polyA$^+$ mRNA from uninduced cells and shows no positively hybridizing band. The positions of molecular mass markers are indicated.

Figure 2 shows the results of expression of recombinant human chondrocyte inducible NO synthase in CHO cells. CHO cells were transfected with pSVL-NO containing the full-length cDNA for human iNOS, and NO synthase activity assayed as NO in the culture supernatant after (A) 24 h or (B) 96 h. Controls were parent cells alone (i.e. untransfected; closed box) and an unrelated CHO-recombinant (pSVLS; hatched box). Only the pSVL-NO recombinant CHO cells produced significant NO in the medium (open box) and this was blocked by incubation with the NO synthase inhibitors L-N-iminoethyl-ornithine L-NIO) (100 μM) and N-guanidino-monomethyl-L-arginine (L-NMMA) (100 μM).

MATERIALS AND METHODS

Cell culture and isolation of mRNA

Human chondrocytes were isolated and cultured. In order to induce NO synthase activity, cells were incubated with IL-1β (1 ng/ml) for 24 h. The methods of culture and induction used were analogous to those described in relation to rabbit chondrocytes by Stadler et al. (1991) J. Immunol 147, 3915-3920 and Palmer et al. (1992) Biochem. Biophys. Res. Commun. 188, 209-215. Cells were harvested with trypsin, washed with growth medium, pelleted and frozen at -70°C. PolyA$^+$ mRNA was extracted with a Micro Fast-Track kit (Trade Name, Invitrogen) from chondrocytes ($1\text{-}2 \times 10^6$ cells) incubated for 24 h with or without IL-1β (1 ng/ml). Typically 1-2 μg polyA$^+$ mRNA was purified from 1×10^6 cells.

The murine macrophage cell line J774 was cultured and induced to express NO synthase with interferon γ and

lipopolysaccharide from Escherichia coli strain 026.B6 as described previously (Cunha et al (1993) J. Immunol 150, 1-6. PolyA$^+$ mRNA was extracted as described above.

Dihydrofolate reductase$^-$ (DHFR$^-$)CHO cells were maintained in 75 cm flasks in Dulbecco's MEM (Trade Name, Gibco), 10% foetal calf serum (FCS), 1 mM L-glutamine, non essential amino acids, antibiotics, 100 μM hypoxanthine and 16 μm thymidine. pSVL transfected cells were cultured in the absence of hypoxanthine and thymidine, but in the presence of dialysed FCS and 100 μM methotrexate. For experimental purposes, cells were trypsinised from the flasks, washed once in phosphate buffered saline (PBS) and plated at 10^6 cells/well in 12 well plates in 3 ml of appropriate culture medium. L-N-iminoethyl-ornithine (L-NIO) or N-guanidino-monomethyl-L-arginine (L-NMMA) was added to some cultures to a final concentration of 100 μM. All cultures were then incubated at 37°C in a humidified 5% CO_2 atmosphere. Samples (100 μl) were removed from triplicate cultures at 24 h intervals and stored at 4°C before determination of NO by chemiluminescence (Palmer et al. (1987), Nature 327, 524-526)

Identification of inos by reverse transcriptase-polymerase chain reaction (RT-PCR) and construction of a cDNA library

The primers BB3: 5'-CGGGATCCGGNACNGGNATHGCNCCNTT-3' (SEQ ID NO: 3) and BB4: 5-GCGAATTCNCCRCANACRTADATRTG-3' (SEQ ID NO: 4) were used to amplify random primed cDNA generated from human induced and uninduced chondrocyte polyA$^+$ mRNA by the polymerase chain reaction (PCR) using a Gene Amp RT-PCR kit (Trade Name, Perkin-Elmer Cetus) following the manufacturers recommended procedures. The following conditions were used: denaturation 96°C, 35s; anneal 55°C, 2 min; and extension 72°C, 3 min for 30 cycles. For a reaction volume of 100 μl, 50 ng of polyA$^+$ mRNA was used with 50 ng of each primer. PCR products were digested with EcoRI-BamHI, resolved by agarose gel electrophoresis, purified and ligated into EcoRI-BamHI digested Bluescript pBS SKII$^+$ (Stratagene) by standard methods (Sambrook

et al. (1989) Molecular cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

RT-PCR was carried out on polyA$^+$ mRNA extracted from induced J774 cells using oligonucleotide primers derived from, the RAW 264.7 sequence (Lyons et al. (1992) J. Biol. Chem. 267 6370-6374; Xie et al. (1992) Science 256, 225-228; and Lowenstein et al. (1992) Proc. Natl. Acad. Sci. USA 89, 6711-6715). The primers, AL14: 5'-ACGGAGAAGCTTAGATCTGGAGCAGAAGTG-3' (SEQ ID NO: 5) and AL15: 5'-CTGCAGGTTGGACCACTGGATCCTGCCGAT-3' (SEQ ID NO: 6) generated a 630 bp band corresponding to the 5-end of the gene. PCR products were digested with *Hind*III and *Bam*HI purified and cloned into *Hind*III-*Bam*HI digested Bluescript pBS SKII+ (Sambrook et al., supra).

PolyA$^+$ mRNA (1.5 μg) isolated from chondrocytes activated with IL-1β (1 ng/ml for 24 h) was used to generate a cDNA library in the bacteriophage lambda ZapII (Stratagene). Both random and oligo-dT primers were used in the cDNA synthesis and 5×10^5 independent recombinant phage were generated. Phage were amplified once, and 10^6 plaques plated out and screened (in duplicate) using standard techniques (Sambrook et al., supra) with the 630 bp fragment from the murine inducible inos gene labelled with [α^{32}P].

Hybridization and DNA sequencing

Blot and plaque hybridizations were carried out on GeneScreen Plus hybridization membranes (Trade Name, DuPont). Northern blot analysis was carried out using digoxigenin labelled probes (Boehringer Mannheim), after electrophoresis and transfer of mRNA from denaturing formaldehyde-agarose gels (Sambrook et al, supra).

Recombinant DNA was sequenced using double standard DNA as template (Stephen et al (1991) Nucleic Acids Res. 24, 7463-7464). An overlapping series of deletions was made in template DNA (Henikoff (1984) Gene 28, 351-359 using the exonuclease III kit (Pharmacia). Sequencing was carried out using universal primer, [α^{35}S] dATP and wedge gels (Sanger et al (1983) Proc. Natl. Acad. Sci. USA 74, 5463-5467). Clones

were sequenced with modified T7 DNA polymerase (Tabor and Richardson (1987) Proc. Natl. Acad. Sci. USA 84, 4767-4771). To resolve compression artifacts (Mizusawa et al. (1986) Nucleic Acids Res. 14, 1319-1324) some clones were sequenced with 7-deaza-2-dGTP (Pharmacia). Gaps in the sequence were filled in using synthetic oligonucleotides made on a Milligen 7500 DNA synthesizer (Trade Name, Millipore) as specific primers (Charles et al (1986) Nucleic Acids. Res, 14, 2201-2213).

Expression of a stable CHO cell line expressing NOS

DHFR⁻ CHO cells were co-transfected with 10 μg of the full-length cDNA for the human inducible NO synthase, pSVL-NO, cloned as an *Xba*I fragment into the vector pSVL (Pharmacia, UK) and with 1 μg of the DHFR encoding plasmid pRDN2 (Dr. N. Sharp, Wellcome Research Laboratories, Beckenham, Kent). Cells were seeded at 10^6 per 100 cm petri dish and individual recombinants cloned by dilution cloning. Individual clones were assayed for the ability to increase the NO_2- concentration in growth medium. One cell line, CHO-INOS-20, expressing the highest levels of inducible NO synthase was selected for further study.

RESULTS

Cloning of an inducible human nos gene

The strategy used to clone the human chondrocyte inducible NO synthase cDNA was based on the finding that significant levels of NO synthase activity can be induced in these cells by IL-1β. Northern blotting showed the presence of a 4.4 kb NOS-specific band in mRNA extracted from induced cells that was absent in uninduced cells (Fig. 1). By using RT-PCR and degenerate oligonucleotide primers, a 350 bp fragment of the rabbit chondrocyte iNOS cDNA was cloned and sequenced which had greater than 90% identity with the murine inducible NO synthase sequence over this region. In order to confirm that the human chondrocyte induction was producing a similar iNOS mRNA to that induced in rabbit chondrocytes, RT-PCR was carried out using the primer set BB3 and BB4. PolyA⁺ mRNA (50 ng) from

induced human cells was used as a template and cDNA was generated by random priming. PCR resulted in a 350 bp band which was purified, cloned and sequenced. Analysis of the sequence demonstrated that the human chondrocyte iNOS cDNA had high (>80%) identity with the murine inducible NOS cDNA over this region.

In order to clone the full-length cDNA for human iNOS, a cDNA library was constructed in lambda ZAPII using oligo dT and random primed polyA$^+$ mRNA isolated from induced cells. To maximize the chance of finding a full-length clone a [α^{32}P]-labelled probe was prepared form a 650 bp 5'-fragment of the murine inducible NOS cDNA cloned from mRNA isolated from the cell line J774. This cell line has a cytokine-inducible NOS cDNA sequence that is identical to that described for the RAW 264.7 cell line (our unpublished observations).

Screening the library with the 5'-probe resulted in the identification of several clones, one of which (pBS HSINOS) contained the full-length cDNA for iNOS. DNA sequence analysis of the 4164 bp cDNA clone showed the presence of an open reading frame capable of encoding a protein of 1153 amino acids with a calculated molecular mass of 131,213 daltons. The start site around, the ATG contains a Kozak consensus sequence (TAGAGATGG; Kozak (1991) J. Cell. Biol. 115, 887-903). Comparison of the deduced sequence of the human inducible NO synthase with its murine counterpart shows the proteins to be highly related. The murine enzyme comprises 1144 amino acids with a calculated molecular mass of 130,556 daltons. Overall, the two proteins have 81% identity and 88% similarity as determined by the GAP align program (Trade Name, Wisconsin GCG). Both molecules have consensus recognition sites for the co-factors FAD, FMN and NADPH and in addition have a calmodulin recognition motif, although both enzymes are Ca^{2+-} independent.

Subcloning of full-length gene as an *Xba*I fragment into the expression vector pSVL generated the construct pSVL-NO. Transfection of this construct into CHO cells led to the isolation of a stable cell line expressing human inducible NO

synthase under the control of a heterologous constitutive promoter (Fig. 2). The expressed NO synthase activity was inhibited by L-NIO (100 μM) and by L-NMMA (100 μM).

Example 2

Description of the Drawings

Figure 3 SDS-PAGE of baculovirus/insect cell expressed human inducible NO synthase.

Tracks 1 and 10, Amersham rainbow molecular weight markers; Track 2, total cell lysate (soluble supernatant fraction;) Tracks 3-9 are fractions from an ADP column eluted with 10mM NADPH. The arrow indicates the position of the 135kDa band corresponding to the iNOS. Samples were run on a 10% SDS-PAGE gel.

MATERIALS AND METHODS

Human inducible NO Synthase CDNA

A full-length human iNOS cDNA fragment was cloned as an *Xba*I fragment into the baculovirus transfer vector pVL1393 to generate pVLHINOS. This vector directs the expression of recombinant proteins under the control of the strong polyhedrin promoter. The human iNOS construct pVLMINOS was used to generate recombinant *Autographa californica* baculovirus using the Baculogold transfection kit (Pharmingen).

Maintenance and infection of Spodoptera frugiperda insect cells

S. frugiperda (Sf-21) cells were maintained as stirred cultures at 27°C in TC100 medium. Roller cultures of Sf-21 cells (5 x 10^8 cells/800cm^2 roller) were infected with human iNOS baculovirus (>10^8pfu/ml) at a ratio of 5pfu/cell for 24 hours at 27°C.

Preparation of NO Synthase

Cytosol preparations containing iNOS were prepared from 2 x 10^9 cells as described below. Briefly, the *S. frugiperda* cell pellet was harvested and washed in a buffer

containing 0.1M Hepes pH7.4, 1.0mM dithiothreitol. Cells were resuspended (10^8/ml) in the same buffer and lysed by 3 freeze-thaw cycles. The resulting lysate was centrifuged at 100,000g for 30 min, and the supernatant mixed at 4°C for 45 mins with one ml of 2'-5' ADP sepharose-4B (Pharmacia), (Charles et al., Biochem. Biophys. Res. Commun., 196, 1481-1489). Following washing, NO synthase activity was eluted from the ADP sepharose with 10mM NADPH (reduced nicotinamide adenine dinuleotide phosphate). Insect cell-derived protein samples wee resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)(Charles et al., Infect. Immun., 59, 1627-1632).

Enzyme Activity

NO sythase activity was measured spectrophotometrically as described previously (Palmer et al., Biochem. Biophys. Res. Commun., 188, 209-215 and Feelisch et al., Eur. J Pharmac., 139, 19-30). In some experiments the effects of altering the arginine concentrations were investigated as was the inhibition of NO synthase with L-NMMA (N^G-monomethyl-L-arginine). The effects of calcium chelation with EGTA (ethylene glycol bis-(β-aminoethylether) N,N,N^1,N^1-tetra acetic acid) and the addition of mammalian calmodulin (bovine, from Sigma) were also determined.

RESULTS

A cDNA clone encoding human inducible NO synthase has been isolated from a λZAPII cDNA library and cloned into the baculovirus expression vector pVL1393 as an *XbaI* fragment. Transfection into *S.frugiperda* (Sf-21) cells results in the expression of NO synthase activity that can be isolated by a freeze-thaw procedure. Fig 3 shows 10% SDS-PAGE gel showing the NADPH elution profile of NO synthase from an ADP sepharose column. Track 2 shows the *S.frugiperda* iNOS cell lysate following high speed centrifugation. No clear band is seen corresponding to recombinant iNOS indicating that the NO synthase is not expressed at high level. This contrasts with

the expression of the neuronal forms of NO synthase that can be expressed at high (15-20% total cell protein) levels in insect cells. Tracks 3-9 show the NADPH elution profile. A major band at 135kDa corresponds to the human iNOS. Lower molecular weight bands seen on the gel cross-react with polyclonal antibody against murine iNOS in western blot experiments (data not shown) suggesting that they represent break-down products of the full-length iNOS.

Kinetic studies on recombinant iNOS shows that it has similar characteristics as its native counterpart, with a similar Km for L-arginine (Table 1). The Vmax measurements are made on crude protein and are solely a measure of enzyme expression, demonstrating that more iNOS is being produced in the baculovirus system than the induced mammalian cells. Inhibition studies using the NO synthase inhibitor L-NMMA demonstrated that recombinant and native iNOS have similar IC_{50} values at 30μM L-arginine, with a Ki for L-NMMA of 15μM. Although chelation of free calcium to a very low concentration by the addition of EGTA (1mM) caused modest inhibition of the recombinant iNOS (59 ± 8.3% inhibition, n=4) this was reversed by the addition of mammalian calmodulin (500μ/ml). Thus, in the presence of mammalian calmodulin the recombinant iNOS was inhibited less than 20% by removal of calcium (18 ± 8.9% inhibition, n=4), consistent with the behaviour of native iNOS (Palmer et al., (1993) Biochem. Biophys. Res. Commun., 193, 398-405; Radomski et al (1991) Cancer Res., 51, 6073-6078; Stuehr et al., (1992) Adv. Enzymol., 65, 287-346).

Table 1: Summary of the characteristics of recombinant human iNOS compared with its native counterpart. The native iNOS was

measured from IL-1 induced human chondrocytes and megakaryocytes (Meg-01) and a human adenocarcinoma cell-line SW480.

Properties of Expressed Recombinant Human iNOS

	Recombinant Human iNOS	Native Human iNOS
Vmax (pmol/min per mg)	430 ± 150	220 (IL-1 treated chondrocytes) 180 (SW480 cells) 3 (IL-1 treated Meg-01 cells)
Km for L-Arg (μM)	4.0 ± 0.38	4 (SW480) 4 (Meg-01)
IC50 for L-NMMA (μM at 30μM L-Arg)	13 ± 2.0	12 (chondrocytes) 19 (Meg-01)

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4164 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(G) CELL TYPE: chondrocyte

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 226..3687

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGAGAACTCA GCCTCATTCC TGCTTTAAAA TCTCTCGGCC ACCTTTGATG AGGGGACTGG 60

GCAGTTCTAG ACAGTCCCGA AGTTCTCAAG GCACAGGTCT CTTCCTGGTT TGACTGTCCT 120

TACCCCGGGG AGGCAGTGCA GCCAGCTGCA AGCCCCACAG TGAAGAACAT CTGAGCTCAA 180

ATCCAGATAA GTGACATAAG TGACCTGCTT TGTAAAGCCA TAGAG ATG GCC TGT 234
Met Ala Cys
1

CCT TGG AAA TTT CTG TTC AAG ACC AAA TTC CAC CAG TAT GCA ATG AAT 282
Pro Trp Lys Phe Leu Phe Lys Thr Lys Phe His Gln Tyr Ala Met Asn
5 10 15

GGG GAA AAA GAC ATC AAC AAC AAT GTG GAG AAA GCC CCC TGT GCC ACC 330
Gly Glu Lys Asp Ile Asn Asn Asn Val Glu Lys Ala Pro Cys Ala Thr
20 25 30 35

TCC AGT CCA GTG ACA CAG GAT GAC CTT CAG TAT CAC AAC CTC AGC AAG 378
Ser Ser Pro Val Thr Gln Asp Asp Leu Gln Tyr His Asn Leu Ser Lys
40 45 50

CAG CAG AAT GAG TCC CCG CAG CCC CTC GTG GAG ACG GGA AAG AAG TCT 426
Gln Gln Asn Glu Ser Pro Gln Pro Leu Val Glu Thr Gly Lys Lys Ser
55 60 65

CCA GAA TCT CTG GTC AAG CTG GAT GCA ACC CCA TTG TCC TCC CCA CGG 474
Pro Glu Ser Leu Val Lys Leu Asp Ala Thr Pro Leu Ser Ser Pro Arg
70 75 80

CAT GTG AGG ATC AAA AAC TGG GGC AGC GGG ATG ACT TTC CAA GAC ACA 522
His Val Arg Ile Lys Asn Trp Gly Ser Gly Met Thr Phe Gln Asp Thr
85 90 95

CTT CAC CAT AAG GCC AAA GGG ATT TTA ACT TGC AGG TCC AAA TCT TGC 570
Leu His His Lys Ala Lys Gly Ile Leu Thr Cys Arg Ser Lys Ser Cys
100 105 110 115

CTG GGG TCC ATT ATG ACT CCC AAA AGT TTG ACC AGA GGA CCC AGG GAC 618
Leu Gly Ser Ile Met Thr Pro Lys Ser Leu Thr Arg Gly Pro Arg Asp
120 125 130

AAG CCT ACC CCT CCA GAT GAG CTT CTA CCT CAA GCT ATC GAA TTT GTC 666
Lys Pro Thr Pro Pro Asp Glu Leu Leu Pro Gln Ala Ile Glu Phe Val
135 140 145

AAC CAA TAT TAC GGC TCC TTC AAA GAG GCA AAA ATA GAG GAA CAT CTG 714
Asn Gln Tyr Tyr Gly Ser Phe Lys Glu Ala Lys Ile Glu Glu His Leu
150 155 160

GCC AGG GTG GAA GCG GTA ACA AAG GAG ATA GAA ACA ACA GGA ACC TAC 762
Ala Arg Val Glu Ala Val Thr Lys Glu Ile Glu Thr Thr Gly Thr Tyr
165 170 175

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Gln Leu Thr Gly Asp Glu Leu Ile Phe Ala Thr Lys Gln Ala Trp Arg
180 185 190 195

AAT GCC CCA CGC TGC ATT GGG AGG ATC CAG TGG TCC AAC CTG CAG GTC 858
Asn Ala Pro Arg Cys Ile Gly Arg Ile Gln Trp Ser Asn Leu Gln Val
200 205 210

TTC GAT GCC CGC AGC TGT TCC ACT GCC CGG GAA ATG TTT GAA CAC ATC 906
Phe Asp Ala Arg Ser Cys Ser Thr Ala Arg Glu Met Phe Glu His Ile
215 220 225

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TGC AGA CAC GTG CGT TAC TCC ACC AAC AAT GGC AAC ATC AGG TCG GCC     954
Cys Arg His Val Arg Tyr Ser Thr Asn Asn Gly Asn Ile Arg Ser Ala
        230                 235                 240

ATC ACC GTG TTC CCC CAG CGG AGT GAT GGC AAG CAC GAC TTC CGG GTG    1002
Ile Thr Val Phe Pro Gln Arg Ser Asp Gly Lys His Asp Phe Arg Val
    245                 250                 255

TGG AAT GCT CAG CTC ATC CGC TAT GCT GGC TAC CAG ATG CCA GAT GGC    1050
Trp Asn Ala Gln Leu Ile Arg Tyr Ala Gly Tyr Gln Met Pro Asp Gly
260                 265                 270                 275

AGC ATC AGA GGG GAC CCT GCC AAC GTG GAA TTC ACT CAG CTG TGC ATC    1098
Ser Ile Arg Gly Asp Pro Ala Asn Val Glu Phe Thr Gln Leu Cys Ile
                280                 285                 290

GAC CTG GGC TGG AAG CCC AAG TAC GGC CGC TTC GAT GTG GTC CCC CTG    1146
Asp Leu Gly Trp Lys Pro Lys Tyr Gly Arg Phe Asp Val Val Pro Leu
            295                 300                 305


GTC CTG CAG GCC AAT GGC CGT GAC CCT GAG CTC TTC GAA ATC CCA CCT    1194
Val Leu Gln Ala Asn Gly Arg Asp Pro Glu Leu Phe Glu Ile Pro Pro
        310                 315                 320

GAC CTT GTG CTT GAG GTG GCC ATG GAA CAT CCC AAA TAC GAG TGG TTT    1242
Asp Leu Val Leu Glu Val Ala Met Glu His Pro Lys Tyr Glu Trp Phe
    325                 330                 335

CGG GAA CTG GAG CTA AAG TGG TAC GCC CTG CCT GCA GTG GCC AAC ATG    1290
Arg Glu Leu Glu Leu Lys Trp Tyr Ala Leu Pro Ala Val Ala Asn Met
340                 345                 350                 355

CTG CTT GAG GTG GGC GGC CTG GAG TTC CCA GGG TGC CCC TTC AAT GGC    1338
Leu Leu Glu Val Gly Gly Leu Glu Phe Pro Gly Cys Pro Phe Asn Gly
                360                 365                 370

TGG TAC ATG GGC ACA GAG ATC GGA GTC CGG GAC TTC TGT GAC GTC CAG    1386
Trp Tyr Met Gly Thr Glu Ile Gly Val Arg Asp Phe Cys Asp Val Gln
            375                 380                 385

CGC TAC AAC ATC CTG GAG GAA GTG GGC AGG AGA ATG GGC CTG GAA ACG    1434
Arg Tyr Asn Ile Leu Glu Glu Val Gly Arg Arg Met Gly Leu Glu Thr
        390                 395                 400

CAC AAG CTG GCC TCG CTC TGG AAA GAC CAG GCT GTC GTT GAG ATC AAC    1482
His Lys Leu Ala Ser Leu Trp Lys Asp Gln Ala Val Val Glu Ile Asn
    405                 410                 415

ATT GCT GTG CTC CAT AGT TTC CAG AAG CAG AAT GTG ACC ATC ATG GAC    1530
Ile Ala Val Leu His Ser Phe Gln Lys Gln Asn Val Thr Ile Met Asp
420                 425                 430                 435

CAC CAC TCG GCT GCA GAA TCC TTC ATG AAG TAC ATG CAG AAT GAA TAC    1578
His His Ser Ala Ala Glu Ser Phe Met Lys Tyr Met Gln Asn Glu Tyr
                440                 445                 450

CGG TCC CGT GGG GGC TGC CCG GCA GAC TGG ATT TGG CTG GTC CCT CCC    1626
Arg Ser Arg Gly Gly Cys Pro Ala Asp Trp Ile Trp Leu Val Pro Pro
            455                 460                 465
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ATG TCT GGG AGC ATC ACC CCC GTG TTT CAC CAG GAG ATG CTG AAC TAC      1674
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GTC CTG TCC CCT TTC TAC TAC TAT CAG GTA GAG GCC TGG AAA ACC CAT      1722
Val Leu Ser Pro Phe Tyr Tyr Tyr Gln Val Glu Ala Trp Lys Thr His
    485                 490                 495

GTC TGG CAG GAC GAG AAG CGG AGA CCC AAG AGA AGA GAG ATT CCA TTG      1770
Val Trp Gln Asp Glu Lys Arg Arg Pro Lys Arg Arg Glu Ile Pro Leu
500                 505                 510                 515

AAA GTC TTG GTC AAA GCT GTG CTC TTT GCC TGT ATG CTG ATG CGC AAG      1818
Lys Val Leu Val Lys Ala Val Leu Phe Ala Cys Met Leu Met Arg Lys
                520                 525                 530

ACA ATG GCG TCC CGA GTC AGA GTC ACC ATC CTC TTT GCG ACA GAG ACA      1866
Thr Met Ala Ser Arg Val Arg Val Thr Ile Leu Phe Ala Thr Glu Thr
            535                 540                 545

GGA AAA TCA GAG GCG CTG GCC TGG GAC CTG GGG GCC TTA TTC AGC TGT      1914
Gly Lys Ser Glu Ala Leu Ala Trp Asp Leu Gly Ala Leu Phe Ser Cys
        550                 555                 560

GCC TTC AAC CCC AAG GTT GTC TGC ATG GAT AAG TAC AGG CTG AGC TGC      1962
Ala Phe Asn Pro Lys Val Val Cys Met Asp Lys Tyr Arg Leu Ser Cys
    565                 570                 575

CTG GAG GAG GAA CGG CTG CTG TTG GTG GTG ACC AGT ACG TTT GGC AAT      2010
Leu Glu Glu Glu Arg Leu Leu Leu Val Val Thr Ser Thr Phe Gly Asn
580                 585                 590                 595

GGA GAC TGC CCT GGC AAT GGA GAG AAA CTG AAG AAA TCG CTC TTC ATG      2058
Gly Asp Cys Pro Gly Asn Gly Glu Lys Leu Lys Lys Ser Leu Phe Met
                600                 605                 610

CTG AAA GAG CTC AAC AAC AAA TTC AGG TAC GCT GTG TTT GGC CTC GGC      2106
Leu Lys Glu Leu Asn Asn Lys Phe Arg Tyr Ala Val Phe Gly Leu Gly
            615                 620                 625

TCC AGC ATG TAC CCT CGG TTC TGC GCC TTT GCT CAT GAC ATT GAT CAG      2154
Ser Ser Met Tyr Pro Arg Phe Cys Ala Phe Ala His Asp Ile Asp Gln
        630                 635                 640

AAG CTG TCC CAC CTG GGG GCC TCT CAG CTC ACC CCG ATG GGA GAA GGG      2202
Lys Leu Ser His Leu Gly Ala Ser Gln Leu Thr Pro Met Gly Glu Gly
    645                 650                 655

GAT GAG CTC AGT GGG CAG GAG GAC GCC TTC CGC AGC TGG GCC GTG CAA      2250
Asp Glu Leu Ser Gly Gln Glu Asp Ala Phe Arg Ser Trp Ala Val Gln
660                 665                 670                 675

ACC TTC AAG GCA GCC TGT GAG ACG TTT GAT GTC CGA GGC AAA CAG CAC      2298
Thr Phe Lys Ala Ala Cys Glu Thr Phe Asp Val Arg Gly Lys Gln His
                680                 685                 690

ATT CAG ATC CCC AAG CTC TAC ACC TCC AAT GTG ACC TGG GAC CCG CAC      2346
Ile Gln Ile Pro Lys Leu Tyr Thr Ser Asn Val Thr Trp Asp Pro His
            695                 700                 705
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CAC TAC AGG CTC GTG CAG GAC TCA CAG CCT TTG GAC CTC AGC AAA GCC      2394
His Tyr Arg Leu Val Gln Asp Ser Gln Pro Leu Asp Leu Ser Lys Ala
        710                 715                 720

CTC AGC AGC ATG CAT GCC AAG AAC GTG TTC ACC ATG AGG CTC AAA TCT      2442
Leu Ser Ser Met His Ala Lys Asn Val Phe Thr Met Arg Leu Lys Ser
    725                 730                 735

CGG CAG AAT CTA CAA AGT CCG ACA TCC AGC CGT GCC ACC ATC CTG GTG      2490
Arg Gln Asn Leu Gln Ser Pro Thr Ser Ser Arg Ala Thr Ile Leu Val
740                 745                 750                 755


GAA CTC TCC TGT GAG GAT GGC CAA GGC CTG AAC TAC CTG CCG GGG GAG      2538
Glu Leu Ser Cys Glu Asp Gly Gln Gly Leu Asn Tyr Leu Pro Gly Glu
                760                 765                 770

CAC CTT GGG GTT TGC CCA GGC AAC CAG CCG GCC CTG GTC CAA GGT ATC      2586
His Leu Gly Val Cys Pro Gly Asn Gln Pro Ala Leu Val Gln Gly Ile
            775                 780                 785

CTG GAG CGA GTG GTG GAT GGC CCC ACA CCC CAC CAG ACA GTG CGC CTG      2634
Leu Glu Arg Val Val Asp Gly Pro Thr Pro His Gln Thr Val Arg Leu
        790                 795                 800

GAG GCC CTG GAT GAG AGT GGC AGC TAC TGG GTC AGT GAC AAG AGG CTG      2682
Glu Ala Leu Asp Glu Ser Gly Ser Tyr Trp Val Ser Asp Lys Arg Leu
    805                 810                 815

CCC CCC TGC TCA CTC AGC CAG GCC CTC ACC TAC TTC CTG GAC ATC ACC      2730
Pro Pro Cys Ser Leu Ser Gln Ala Leu Thr Tyr Phe Leu Asp Ile Thr
820                 825                 830                 835

ACA CCC CCA ACC CAG CTG CTG CTC CAA AAG CTG GCC CAG GTG GCC ACA      2778
Thr Pro Pro Thr Gln Leu Leu Leu Gln Lys Leu Ala Gln Val Ala Thr
                840                 845                 850

GAA GAG CCT GAG AGA CAG AGG CTG GAG GCC CTG TGC CAG CCC TCA GAG      2826
Glu Glu Pro Glu Arg Gln Arg Leu Glu Ala Leu Cys Gln Pro Ser Glu
            855                 860                 865

TAC AGC AAG TGG AAG TTC ACC AAC AGC CCC ACA TTC CTG GAG GTG CTA      2874
Tyr Ser Lys Trp Lys Phe Thr Asn Ser Pro Thr Phe Leu Glu Val Leu
        870                 875                 880

GAG GAG TTC CCG TCC CTG CGG GTG TCT GCT GGC TTC CTG CTT TCC CAG      2922
Glu Glu Phe Pro Ser Leu Arg Val Ser Ala Gly Phe Leu Leu Ser Gln
    885                 890                 895

CTC CCC ATT CTG AAG CCC AGG TTC TAC TCC ATC AGC TCC TCC CGG GAT      2970
Leu Pro Ile Leu Lys Pro Arg Phe Tyr Ser Ile Ser Ser Ser Arg Asp
900                 905                 910                 915
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CAC ACG CCC ACA GAG ATC CAC CTG ACT GTG GCC GTG GTC ACC TAC CAC 3018
His Thr Pro Thr Glu Ile His Leu Thr Val Ala Val Val Thr Tyr His
920 925 930

ACC CGA GAT GGC CAG GGT CCC CTG CAC CAC GGC GTC TGC AGC ACA TGG 3066
Thr Arg Asp Gly Gln Gly Pro Leu His His Gly Val Cys Ser Thr Trp
935 940 945

CTC AAC AGC CTG AAG CCC CAA GAC CCA GTG CCC TGC TTT GTG CGG AAT 3114
Leu Asn Ser Leu Lys Pro Gln Asp Pro Val Pro Cys Phe Val Arg Asn
950 955 960

GCC AGC GGC TTC CAC CTC CCC GAG GAT CCC TCC CAT CCT TGC ATC CTC 3162
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965 970 975

ATC GGG CCT GGC ACA GGC ATC GCG CCC TTC CGC AGT TTC TGG CAG CAA 3210
Ile Gly Pro Gly Thr Gly Ile Ala Pro Phe Arg Ser Phe Trp Gln Gln
980 985 990 995

CGG CTC CAT GAC TCC CAG CAC AAG GGA GTG CGG GGA GGC CGC ATG ACC 3258
Arg Leu His Asp Ser Gln His Lys Gly Val Arg Gly Gly Arg Met Thr
1000 1005 1010

TTG GTG TTT GGG TGC CGC CGC CCA GAT GAG GAC CAC ATC TAC CAG GAG 3306
Leu Val Phe Gly Cys Arg Arg Pro Asp Glu Asp His Ile Tyr Gln Glu
1015 1020 1025

GAG ATG CTG GAG ATG GCC CAG AAG GGG GTG CTG CAT GCG GTG CAC ACA 3354
Glu Met Leu Glu Met Ala Gln Lys Gly Val Leu His Ala Val His Thr
1030 1035 1040

GCC TAT TCC CGC CTG CCT GGC AAG CCC AAG GTC TAT GTT CAG GAC ATC 3402
Ala Tyr Ser Arg Leu Pro Gly Lys Pro Lys Val Tyr Val Gln Asp Ile
1045 1050 1055

CTG CGG CAG CAG CTG GCC AGC GAG GTG CTC CGT GTG CTC CAC AAG GAG 3450
Leu Arg Gln Gln Leu Ala Ser Glu Val Leu Arg Val Leu His Lys Glu
1060 1065 1070 1075

CCA GGC CAC CTC TAT GTT TGC GGG GAT GTG CGC ATG GCC CGG GAC GTG 3498
Pro Gly His Leu Tyr Val Cys Gly Asp Val Arg Met Ala Arg Asp Val
1080 1085 1090

GCC CAC ACC CTG AAG CAG CTG GTG GCT GCC AAG CTG AAA TTG AAT GAG 3546
Ala His Thr Leu Lys Gln Leu Val Ala Ala Lys Leu Lys Leu Asn Glu
1095 1100 1105

GAG CAG GTC GAG GAC TAT TTC TTT CAG CTC AAG AGC CAG AAG CGC TAT 3594
Glu Gln Val Glu Asp Tyr Phe Phe Gln Leu Lys Ser Gln Lys Arg Tyr
1110 1115 1120

CAC GAA GAT ATC TTT GGT GCT GTA TTT CCT TAC GAG GCG AAG AAG GAC 3642
His Glu Asp Ile Phe Gly Ala Val Phe Pro Tyr Glu Ala Lys Lys Asp
1125 1130 1135

AGG GTG GCG GTG CAG CCC AGC AGC CTG GAG ATG TCA GCG CTC TGAGGGCCTA 3694
Arg Val Ala Val Gln Pro Ser Ser Leu Glu Met Ser Ala Leu
1140 1145 1150

CAGGAGGGGT TAAAGCTGCC GGCACAGAAC TTAAGGATGG AGCCAGCTCT GCATTATCTG 3754

AGGTCACAGG GCCTGGGGAG ATGGAGGAAA GTGATATCCC CCAGCCTCAA GTCTTATTTC 3814

CTCAACGTTG CTCCCCATCA AGCCCTTTAC TTGACCTCCT AACAAGTAGC ACCCTGGATT 3874

GATCGGAGCC TCCTCTCTCA AACTGGGGCC TCCCTGGTCC CTTGGAGACA AAATCTTAAA 3934

TGCCAGGCCT GGCAAGTGGG TGAAAGATGG AACTTGCTGC TGAGTGCACC ACTTCAAGTG 3994

ACCACCAGGA GGTGCTATCG CACCACTGTG TATTTAACTG CCTTGTGTAC AGTTATTTAT 4054

GCCTCTGTAT TTAAAAAACT AACACCCAGT CTGTTCCCCA TGGCCACTTG GGTCTTCCCT 4114

GTATGATTCC TTGATGGAGA TATTTACATG AATTGCATTT TACTTTAATC 4164

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1153 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Ala Cys Pro Trp Lys Phe Leu Phe Lys Thr Lys Phe His Gln Tyr
 1               5                  10                  15

Ala Met Asn Gly Glu Lys Asp Ile Asn Asn Asn Val Glu Lys Ala Pro
            20                  25                  30

Cys Ala Thr Ser Ser Pro Val Thr Gln Asp Asp Leu Gln Tyr His Asn
        35                  40                  45

Leu Ser Lys Gln Gln Asn Glu Ser Pro Gln Pro Leu Val Glu Thr Gly
    50                  55                  60

Lys Lys Ser Pro Glu Ser Leu Val Lys Leu Asp Ala Thr Pro Leu Ser
65                  70                  75                  80

Ser Pro Arg His Val Arg Ile Lys Asn Trp Gly Ser Gly Met Thr Phe
                85                  90                  95

Gln Asp Thr Leu His His Lys Ala Lys Gly Ile Leu Thr Cys Arg Ser
            100                 105                 110

Lys Ser Cys Leu Gly Ser Ile Met Thr Pro Lys Ser Leu Thr Arg Gly
        115                 120                 125

Pro Arg Asp Lys Pro Thr Pro Pro Asp Glu Leu Leu Pro Gln Ala Ile
    130                 135                 140

Glu Phe Val Asn Gln Tyr Tyr Gly Ser Phe Lys Glu Ala Lys Ile Glu
145                 150                 155                 160

Glu His Leu Ala Arg Val Glu Ala Val Thr Lys Glu Ile Glu Thr Thr
                165                 170                 175
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Gly Thr Tyr Gln Leu Thr Gly Asp Glu Leu Ile Phe Ala Thr Lys Gln
            180                 185                 190

Ala Trp Arg Asn Ala Pro Arg Cys Ile Gly Arg Ile Gln Trp Ser Asn
        195                 200                 205

Leu Gln Val Phe Asp Ala Arg Ser Cys Ser Thr Ala Arg Glu Met Phe
    210                 215                 220

Glu His Ile Cys Arg His Val Arg Tyr Ser Thr Asn Asn Gly Asn Ile
225                 230                 235                     240

Arg Ser Ala Ile Thr Val Phe Pro Gln Arg Ser Asp Gly Lys His Asp
                245                 250                 255

Phe Arg Val Trp Asn Ala Gln Leu Ile Arg Tyr Ala Gly Tyr Gln Met
            260                 265                 270

Pro Asp Gly Ser Ile Arg Gly Asp Pro Ala Asn Val Glu Phe Thr Gln
        275                 280                 285

Leu Cys Ile Asp Leu Gly Trp Lys Pro Lys Tyr Gly Arg Phe Asp Val
    290                 295                 300

Val Pro Leu Val Leu Gln Ala Asn Gly Arg Asp Pro Glu Leu Phe Glu
305                 310                 315                     320

Ile Pro Pro Asp Leu Val Leu Glu Val Ala Met Glu His Pro Lys Tyr
                325                 330                 335

Glu Trp Phe Arg Glu Leu Glu Leu Lys Trp Tyr Ala Leu Pro Ala Val
            340                 345                 350

Ala Asn Met Leu Leu Glu Val Gly Gly Leu Glu Phe Pro Gly Cys Pro
        355                 360                 365

Phe Asn Gly Trp Tyr Met Gly Thr Glu Ile Gly Val Arg Asp Phe Cys
    370                 375                 380

Asp Val Gln Arg Tyr Asn Ile Leu Glu Glu Val Gly Arg Arg Met Gly
385                 390                 395                     400

Leu Glu Thr His Lys Leu Ala Ser Leu Trp Lys Asp Gln Ala Val Val
                405                 410                 415

Glu Ile Asn Ile Ala Val Leu His Ser Phe Gln Lys Gln Asn Val Thr
            420                 425                 430

Ile Met Asp His His Ser Ala Ala Glu Ser Phe Met Lys Tyr Met Gln
        435                 440                 445

Asn Glu Tyr Arg Ser Arg Gly Gly Cys Pro Ala Asp Trp Ile Trp Leu
    450                 455                 460

Val Pro Pro Met Ser Gly Ser Ile Thr Pro Val Phe His Gln Glu Met
465                 470                 475                     480

Leu Asn Tyr Val Leu Ser Pro Phe Tyr Tyr Tyr Gln Val Glu Ala Trp
                485                 490                 495

Lys Thr His Val Trp Gln Asp Glu Lys Arg Arg Pro Lys Arg Arg Glu
            500                 505                 510
```

Ile Pro Leu Lys Val Leu Val Lys Ala Val Leu Phe Ala Cys Met Leu
        515   520   525

Met Arg Lys Thr Met Ala Ser Arg Val Arg Val Thr Ile Leu Phe Ala
    530   535   540

Thr Glu Thr Gly Lys Ser Glu Ala Leu Ala Trp Asp Leu Gly Ala Leu
545   550   555   560

Phe Ser Cys Ala Phe Asn Pro Lys Val Val Cys Met Asp Lys Tyr Arg
                565   570   575

Leu Ser Cys Leu Glu Glu Glu Arg Leu Leu Leu Val Val Thr Ser Thr
            580   585   590

Phe Gly Asn Gly Asp Cys Pro Gly Asn Gly Glu Lys Leu Lys Lys Ser
        595   600   605

Leu Phe Met Leu Lys Glu Leu Asn Asn Lys Phe Arg Tyr Ala Val Phe
    610   615   620

Gly Leu Gly Ser Ser Met Tyr Pro Arg Phe Cys Ala Phe Ala His Asp
625   630   635   640

Ile Asp Gln Lys Leu Ser His Leu Gly Ala Ser Gln Leu Thr Pro Met
                645   650   655

Gly Glu Gly Asp Glu Leu Ser Gly Gln Glu Asp Ala Phe Arg Ser Trp
            660   665   670

Ala Val Gln Thr Phe Lys Ala Ala Cys Glu Thr Phe Asp Val Arg Gly
        675   680   685

Lys Gln His Ile Gln Ile Pro Lys Leu Tyr Thr Ser Asn Val Thr Trp
    690   695   700

Asp Pro His His Tyr Arg Leu Val Gln Asp Ser Gln Pro Leu Asp Leu
705   710   715   720

Ser Lys Ala Leu Ser Ser Met His Ala Lys Asn Val Phe Thr Met Arg
                725   730   735

Leu Lys Ser Arg Gln Asn Leu Gln Ser Pro Thr Ser Ser Arg Ala Thr
            740   745   750

Ile Leu Val Glu Leu Ser Cys Glu Asp Gly Gln Gly Leu Asn Tyr Leu
        755   760   765

Pro Gly Glu His Leu Gly Val Cys Pro Gly Asn Gln Pro Ala Leu Val
    770   775   780

Gln Gly Ile Leu Glu Arg Val Val Asp Gly Pro Thr Pro His Gln Thr
785   790   795   800

Val Arg Leu Glu Ala Leu Asp Glu Ser Gly Ser Tyr Trp Val Ser Asp
                805   810   815

Lys Arg Leu Pro Pro Cys Ser Leu Ser Gln Ala Leu Thr Tyr Phe Leu
            820   825   830

Asp Ile Thr Thr Pro Pro Thr Gln Leu Leu Leu Gln Lys Leu Ala Gln
835 840 845

Val Ala Thr Glu Glu Pro Glu Arg Gln Arg Leu Glu Ala Leu Cys Gln
850 855 860

Pro Ser Glu Tyr Ser Lys Trp Lys Phe Thr Asn Ser Pro Thr Phe Leu
865 870 875 880

Glu Val Leu Glu Glu Phe Pro Ser Leu Arg Val Ser Ala Gly Phe Leu
885 890 895

Leu Ser Gln Leu Pro Ile Leu Lys Pro Arg Phe Tyr Ser Ile Ser Ser
900 905 910

Ser Arg Asp His Thr Pro Thr Glu Ile His Leu Thr Val Ala Val Val
915 920 925

Thr Tyr His Thr Arg Asp Gly Gln Gly Pro Leu His His Gly Val Cys
930 935 940

Ser Thr Trp Leu Asn Ser Leu Lys Pro Gln Asp Pro Val Pro Cys Phe
945 950 955 960

Val Arg Asn Ala Ser Gly Phe His Leu Pro Glu Asp Pro Ser His Pro
965 970 975

Cys Ile Leu Ile Gly Pro Gly Thr Gly Ile Ala Pro Phe Arg Ser Phe
980 985 990

Trp Gln Gln Arg Leu His Asp Ser Gln His Lys Gly Val Arg Gly Gly
995 1000 1005

Arg Met Thr Leu Val Phe Gly Cys Arg Arg Pro Asp Glu Asp His Ile
1010 1015 1020

Tyr Gln Glu Glu Met Leu Glu Met Ala Gln Lys Gly Val Leu His Ala
1025 1030 1035 1040

Val His Thr Ala Tyr Ser Arg Leu Pro Gly Lys Pro Lys Val Tyr Val
1045 1050 1055

Gln Asp Ile Leu Arg Gln Gln Leu Ala Ser Glu Val Leu Arg Val Leu
1060 1065 1070

His Lys Glu Pro Gly His Leu Tyr Val Cys Gly Asp Val Arg Met Ala
1075 1080 1085

Arg Asp Val Ala His Thr Leu Lys Gln Leu Val Ala Ala Lys Leu Lys
1090 1095 1100

Leu Asn Glu Glu Gln Val Glu Asp Tyr Phe Phe Gln Leu Lys Ser Gln
1105 1110 1115 1120

Lys Arg Tyr His Glu Asp Ile Phe Gly Ala Val Phe Pro Tyr Glu Ala
1125 1130 1135

Lys Lys Asp Arg Val Ala Val Gln Pro Ser Ser Leu Glu Met Ser Ala
1140 1145 1150

Leu

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGGATCCGG NACNGGNATH GCNCCNTT 28

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCGAATTCNC CRCANACRTA DATRTG 26

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACGGAGAAGC TTAGATCTGG AGCAGAAGTG 30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGCAGGTTG GACCACTGGA TCCTGCCGAT 30

CLAIMS

1. A nitric oxide (NO) synthase having a sequence at least 85% identical to the sequence of SEQ ID NO: 2.

2. A NO synthase according to Claim 1 having a sequence at least 90% identical to the sequence of SEQ ID NO:2.

3. A NO synthase according to Claim 2 having a sequence at least 95% identical to the sequence of SEQ ID NO:2.

4. A NO synthase according to Claim 3 having a sequence at least 99% identical to the sequence of SEQ ID NO:2.

5. An NO synthase according to claim any one of claims 1 to 4 which is of human origin.

6. An NO synthase according to any preceeding claim in substantially pure form.

7. A DNA molecule encoding an NO synthase according to any preceeding claim.

8. A replicable expression vector containing a DNA molecule according to Claim 7.

9. A host cell transformed or transfected with a vector as claimed in claim 8.

10. A method of producing an NO synthase according to any one of claims 1 to 6, which comprises

(a) culturing a host cell according to claim 9 under conditions in which the cell expresses the NO synthase; and

(b) recovering the NO synthase from the culture.

11. An antisense oligonucleotide having a sequence at least 85% identical to the sequence of ID NO:1.

12. An antisense oligonucleotide having a sequence at least 95% identical to the sequence of ID NO:1.

13. An antisense oligonucleotide according to either of claims 11 and 12 in which the fragment is 12 to 30 nucleotides in length.

14. An antibody specific for an NO synthase according to any one of claims 1 to 6.

15. A method of detecting or quantitatively determining in a sample an NO synthase according to any one of claims 1 to 6, which comprises

(a) contacting the sample with an antibody as claimed in claim 14; and

(b) detecting or quantitatively determining the binding of the antibody.

16. A method for identifying a substrate which inhibits or stimulates an NO synthase according to any one of claims 1 to 6, which comprises

(a) incubating the NO synthase with the substrate;

(b) measuring the activity of the NO synthase; and

(c) comparing the activity measured in (b) above with the activity of the NO synthase in the absence of the substrate.

17. A substrate identified by method according to claim 16.

18. An enzyme-substrate complex which comprises an NO synthase according to any of claims 1 to 6 and a substrate according to Claim 17.

19. A pharmaceutical formulation which comprises one or more of a substrate according to Claim 17, a NO synthase according to any one of Claims 1 to 6 or an antibody according to Claim 14, in combination with a pharmaceutically acceptable carrier or diluent therefor, and optionally one or more further therapeutic agents.

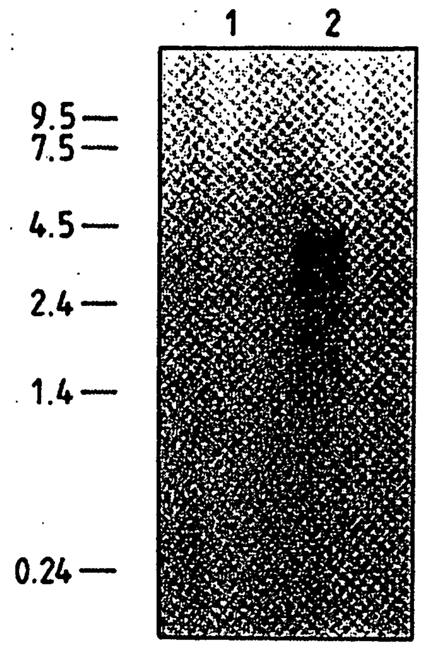

Fig. 1

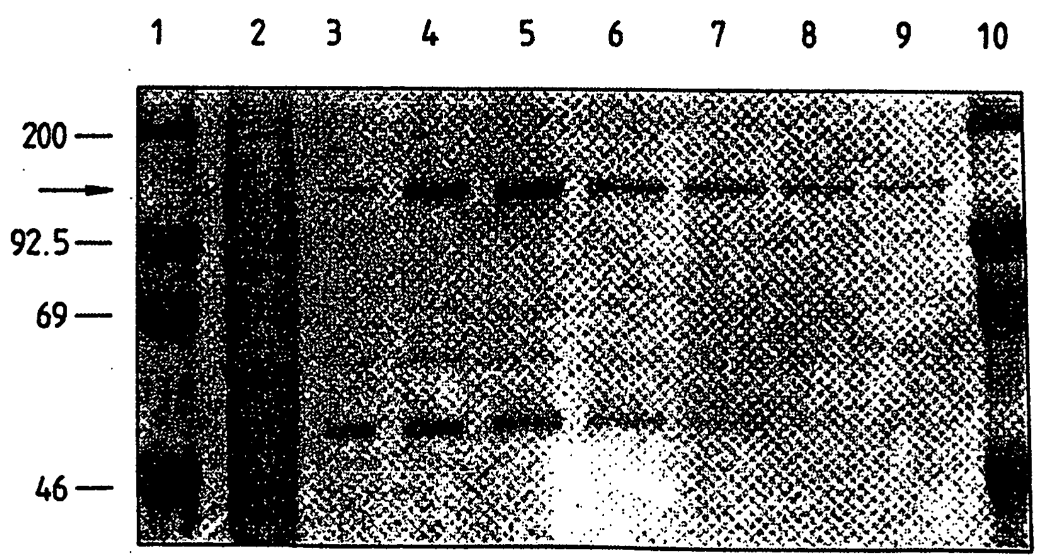

Fig. 3

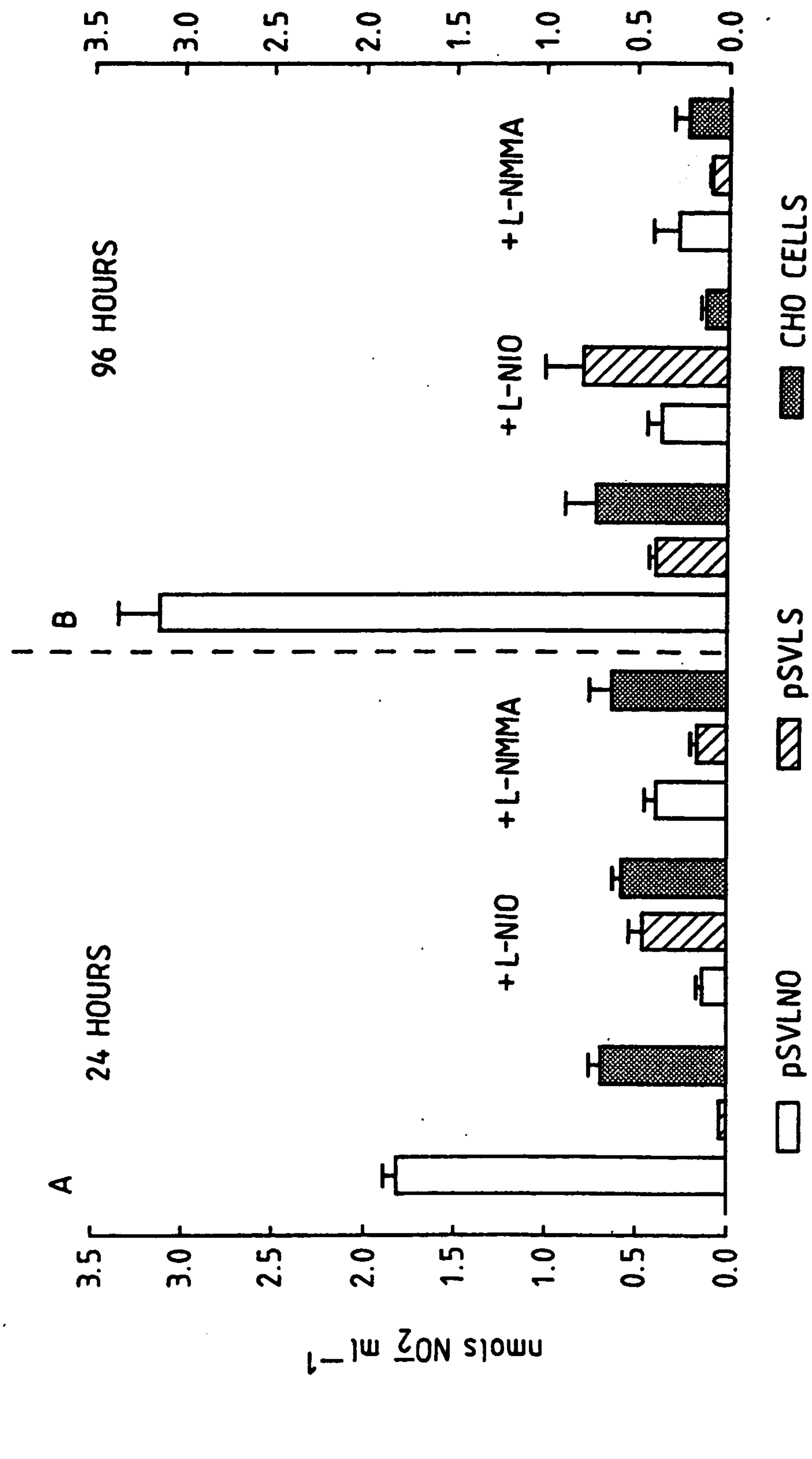
A
B
24 HOURS
96 HOURS
+L-NIO
+L-NMMA
+L-NIO
+L-NMMA
nmols NO_2^- ml^{-1}
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
pSVLNO
pSVLS
CHO CELLS

Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/00621

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/53 C12N15/85 C12N9/02 C07K15/06 A61K39/395
A61K37/50 C12Q1/527 C12Q1/68 G01N33/573

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. BIOL. CHEM. vol. 267, no. 9 , 25 March 1992 pages 6370 - 6374 LYONS ET AL. 'Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line' cited in the application ---	1,6-11, 13
X	BIOCHEM. BIOPHYS. RES. COMMUN. vol. 191, no. 1 , 26 February 1993 pages 89 - 94 NUNOKAWA ET AL. 'Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells' ---	1,6,7, 11,13,19

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

Date of the actual completion of the international search	Date of mailing of the international search report
29 July 1994	- 4. 08. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gac, G

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROC. NATL ACAD. SCI. vol. 89 , 1 August 1992 pages 6711 - 6715 LOWENSTEIN ET AL. 'Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme' cited in the application ---	1,6-11
P,X	PROC. NATL ACAD. SCI. vol. 90 , December 1993 pages 11419 - 11423 CHARLES ET AL. 'Cloning, characterization and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte' ---	1-13
P,X	ARTHRIT. RHEUMAT. vol. 36, no. SUP9 , September 1993 page S189 REDICKE ET AL. 'Human articular chondrocytes induced by proinflammatory cytokines are the major intraarticular source of nitric oxide (NO) and NO mediates the IL-1 induction of cGMP' -----	1-6, 16-19

3